REMARKS

In order to more particularly point out and distinctly claim the present invention, applicants have amended claims 1, 2, 5-8, 10, and 24. These claims have been amended in accordance with the Examiner's suggestions. Moreover, claim 2 has been amended to recite the specific amino acid sequence of SEQ ID NO:2. Support for this amendment is found at page 45, lines 7-25 of the present specification. Claims 5-7 have been amended to more clearly recite the terms "derivatives" and "fragmentary, contiguous nucleic acid sequence." Support for these amendments are found at page 52, lines 21-24 and page 51, lines 3-6 of the present specification, respectively. The descriptive portion of the present specification has been amended to improve the syntax of the specification. In view of the above, it is believed to be apparent that the present amendment introduces no new matter into the present specification.

In the outstanding Official Action, the specification was objected to because the priority data is not recited in the first line of the specification. In the interest of advancing prosecution, applicants have amended the present specification to recite the priority data as suggested by the Examiner.

Claims 8, 10, and 24 were objected to for containing several informalities. The Official Action suggested that the following amendments would obviate the objections pertaining to

claims 8, 10, and 24:

1) amend the claim to recite 'any one of claims 3, 4, 5 or 6';

2) replace the word 'obtainable' with 'prepared'; and

3) remove the second recitation of 'or the peptide of claim 2'.

In order to remove these objections, applicants have amended claims 8, 10, and 24 in accordance with the Examiner's suggestions. Thus, it is believed that the objections to claims 8, 10, and 24 have been obviated, and applicants would like to thank Examiner Landsman for his help on this matter.

In the outstanding Official Action, claims 5-9 were rejected under 35 USC §112, first paragraph, for allegedly being based on a non-enabling disclosure and an insufficient written description.

The Official Action alleged that while the present disclosure is enabling for the isolated DNA of SEQ ID NO:3 and 4, the present disclosure does not reasonably provide enablement for "a derivative thereof" of SEQ ID NO:3 or 4, or DNA which encodes at least 12 contiguous nucleotides of SEQ ID NO:3 or 4, or peptides comprising at least 5 contiguous residues of SEQ ID NO:2.

Claims 5-9 were then further rejected under 35 USC §112, first paragraph, for allegedly containing subject matter which was not described in the specification in such a way as to reasonably convey to one skilled in the relevant art that the inventors, at the time the application was filed, had possession of the claimed invention. Specifically, the Official Action alleges that "nucleic acid molecules which are 'derivatives' of SEQ ID NO: 3 or 4 would have one or more nucleic acid substitutions, deletions, insertions and/or additions to said polynucleotides" and "the scope of the claims includes numerous structural variants".

These rejections are respectfully traversed.

Applicants have amended claims 5, 6 and 7 by deleting "derivatives" and inserting "chemically modified nucleic acid derivatives". This amendment is supported by the description found at page 52, lines 21-24 of the present specification. In fact, the present specification provides explicit examples of nucleic acid derivatives. For example, the present specification recites "methylated, methyl phosphorylated, deaminated or thiophosphorylated nucleic acid derivatives".

All of these derivatives are chemically modified nucleic acid derivatives obtained by chemically modifying a base nucleotide sequence, which is a fragmentary sequence of at least 20 contiguous nucleotides in the DNA sequence of SEQ ID NO: 3 or

4, or an RNA sequence which is complementary to the nucleotide sequence of SEQ ID NO:3. The Examiner's attention is also respectfully directed to the article, "ANTISENSE RNA AND DNA", Murray, J. A. H. ed., Wiley-Liss, Inc., 1992, pp. 1-14. The article is enclosed with this amendment as Exhibit 1 and described at page 54, lines 9-10 in the present specification.

According to Exhibit 1, an oligonucleotide derivative is an oligonucleotide having a modified linkage or terminal group which has been prepared by using the techniques of synthetic organic chemistry (see page 8, left-hand column, lines 36-41 of Exhibit 1). Thus, it is believed that the chemically modified nucleic acid derivatives of the present invention are clearly set forth in the present disclosure so that one of ordinary skill in the art would be able to practice the claimed invention.

Moreover, while the Official Action contends that applicants do not provide the function of the derivatives with the encoded protein, applicants believe that the function of these derivatives and proteins are clearly stated.

The DNA fragments, RNA fragments and fragment derivatives thereof can be used as primers, probes or as antisense DNA or antisense RNA. They are also useful for detecting cDNA or genomic DNA of a protein of the present invention (see page 50, line 24 to page 52, line 7). They can be used for examining the expression of genes relating to the

present invention (see page 52, line 8 to page 53, line 3), detecting homologues of these genes (see page 53, lines 4-19), and elucidating specific functions of protein pertaining to the present invention (see page 53, line 20 to page 54, line 10). Moreover, a fragmentary peptide encoded by a DNA fragment, RNA fragment or a derivative thereof of the present invention can be used as an antigen for producing an antibody. Specific examples of these fragmentary peptides are described at page 45, lines 7-25 in the present specification.

Thus, in view of the above, it is believed that the claimed invention is supported by an enabling disclosure and sufficient written description.

Claims 1-4, 8, 10-12, 23 and 24 were rejected under 35 USC §112, second paragraph, as allegedly being indefinite for failing to particularly point out and distinctly claim the subject matter which applicants regard as their invention. This rejection is traversed.

In order to more particularly point out and distinctly claim the present invention, applicants have amended claims 1 and 2 by changing "substantially pure" to "purified". This amendment is supported by the description found in Example 5 of the present specification. Applicants believe that the indefiniteness rejection of claims 1 and 2 has been obviated by the present amendment.

Claims 2, 23 and 24 were rejected under 35 USC §102(b) as allegedly being anticipated by LI et al. 5,861,272. This rejection is traversed.

Specifically, the Official Action contends:

> "The claims recite a peptide comprising at least 5 contiguous amino acids of SEQ ID NO:2, vectors, host cells and methods of screening for ligands which bind to said receptor. Li et al. teach a peptide comprising 5 contiguous amino acids of SEQ ID NO:2 (Sequence Comparison A). Li et al. also teach vectors and host cells (column 7, line 37-column 10, line 52; column 20, line 9-column 22, line 38) and screening for ligands which bind said receptor (column 10, line 53-column 12, line 52)."

Applicants have amended claim 2 to recite a "fragmentary, contiguous amino acid sequence" for the following amino acid sequences: the 6th to 32nd amino acid residues of SEQ ID NO:2, the 1st to 23rd amino acid residues of SEQ ID NO:2, the 1st to 35th amino acid residues of SEQ ID NO:2, the 96th to 108th amino acid residues of SEQ ID NO:2, the 172nd to 198th amino acid residues of SEQ ID NO:2 and the 681st to 726th amino acid residues of SEQ ID NO:2. Support for this amendment is found at page 45, lines 7-25 of the present specification.

As apparent from Sequence Comparison A, LI et al. teach a peptide comprising at least 5 contiguous amino acids of SEQ ID NO:2. However, LI et al. fail to teach or suggest the above-mentioned specific peptides each containing more than 20 contiguous amino acids of SEQ ID NO:2. Thus, applicants believe

that the rejection of claim 2 has been obviated by the present amendment.

Claims 5 and 7 were rejected under 35 USC §102(b) as allegedly being anticipated by ALVAREZ et al. (Immunogenetics 44(6):446-452, 1996). Claim 6 was further rejected under 35 USC §102(b) as allegedly anticipated by ALVAREZ et al. These rejections are traversed.

Applicants have amended claims 5, 6 and 7 to recite "at least 12 nucleotides" rather than "at least 20 nucleotides". This amendment is supported by the description found at page 51, lines 3-6 of the present specification.

As apparent from Sequence Comparisons B and C, at best, ALVAREZ et al. teach a DNA comprising 17 nucleotides of SEQ ID NO:3. However, ALVAREZ et al. fail to disclose or suggest a nucleotide sequence comprising 20 nucleotides of SEQ ID NO:3. Thus, applicants believe that ALVAREZ et al. fail to anticipate or render obvious the claimed invention.

Claims 8-10 were rejected under 35 USC §103(a) as allegedly being unpatentable over ALVAREZ et al. in view of SIBSON et al. This rejection is traversed.

The Official Action alleges the following:

> "The claims recite a vector comprising a fragment of SEQ ID NO:3 or 4, a host cell and a method of making a protein. Alvarez et al. teach a DNA comprising 17 contiguous nucleotides of SEQ ID No:3 (Sequence Comparison B) and 4 (Sequence Comparison C). Alvarez et al. do not teach vectors, host cells and a methods for making the protein. However, Sibson et al. do teach

expression vectors, host cells and a method of making the protein (page 7, line 39-page 9, line 10)."

Applicants have amended claims 5 and 6 by changing "at least 12 nucleotides" to "at least 20 nucleotides". This amendment is supported by the description found at page 51, lines 3-6 of the present specification. By way of the present amendment, the DNA fragment of claim 8 recites a fragmentary sequence of at least 20 contiguous nucleotides of SEQ ID NO:3 or 4.

As noted above, ALVAREZ et al. teach only a DNA comprising 17 contiguous nucleotides of SEQ ID NO:3 or 4, and ALVAREZ et al. do not teach a fragmentary nucleotide sequence comprising 20 contiguous nucleotides of SEQ ID NO:3 or 4. Therefore, it is believed that ALVAREZ et al., alone or taken in combination with SIBSON et al., fail to disclose or suggest the claimed invention.

With respect to claim 10, applicants note that the DNA used to produce the seven-pass transmembrane receptor protein of the present invention is "the isolated DNA according to claim 3 or 4", namely "an isolated DNA encoding the seven-pass transmembrane receptor protein of claim 1". Thus, the DNA used to produce the protein of claim 10 is a whole length DNA encoding the seven-pass transmembrane receptor protein, and not a fragmentary DNA. Therefore, it cannot be said that ALVAREZ et

al. in view of SIBSON et al. suggest the production of the claimed seven-pass transmembrane receptor protein by use of a whole length DNA encoding the protein of claim 1.

From the forgoing, it is believed that the rejections of the Official Action have been obviated. Applicants believe that the present application is now in condition for allowance.

Attached hereto is a marked-up version of the changes made to the specification and claims. The attached page is captioned "VERSION WITH MARKINGS TO SHOW CHANGES MADE."

Respectfully submitted,

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April 9, 2003

VERSION WITH MARKINGS TO SHOW CHANGES MADE

IN THE SPECIFICATION:

Page 23, the paragraph beginning on line 21 has been amended as follows:

--2. A [substantially pure] purified peptide which is a fragmentary[, contiguous] sequence [of at least 5 amino acids in the amino acid sequence of SEQ ID NO:2] selected from the group consisting of the 6th to 32nd amino acid residues of SEQ ID NO:2, the 1st to 23rd amino acid residues of SEQ ID NO:2, the 1st to 35th amino acid residues of SEQ ID NO:2, the 96th to 108th amino acid residues of SEQ ID NO:2, the 172nd to 198th amino acid residues of SEQ ID NO:2, and the 681st to 726th amino acid residues of SEQ ID NO:2.--.

Page 24, the paragraph beginning on line 6 has been amended as follows:

--5. An isolated DNA or a chemically modified nucleic acid derivative thereof, wherein the isolated DNA is a fragmentary[, contiguous] sequence of at least [12] 20 contiguous nucleotides in the nucleotide sequence of SEQ ID NO:3.--;

Page 24, the paragraph beginning on line 11 has been amended as follows:

--6. An isolated DNA or a chemically modified nucleic acid derivative thereof, wherein the isolated DNA is a fragmentary[, contiguous] sequence of at least [12] 20 contiguous nucleotides

in the nucleotide sequence of SEQ ID NO:4.--;

Page 24, the paragraph beginning on line 16 has been amended as follows:

--7. An isolated RNA or a chemically modified nucleic acid derivative thereof, wherein the isolated RNA is a fragmentary[, contiguous] sequence of at least [12] 20 contiguous nucleotides in an RNA which is complementary to the nucleotide sequence of SEQ ID NO:3.--.

Page 44, the paragraph beginning on line 16 and bridging pages 44 and 45 has been amended as follows:

--The C5L2 protein and the fragments thereof are useful for producing an antibody for use in diagnosis and useful for screening pharmaceuticals for treating diseases. Each of the above-mentioned fragments is a fragmentary peptide having a part of the amino acid sequence of C5L2 protein, specifically a peptide which is a fragmentary[, contiguous] sequence of at least 5 contiguous amino acids in the whole C5L2 protein. Like the whole protein, such a fragmentary peptide is useful for the production of an antibody, the screening of a ligand, and the detection of a substance which binds to C5L2 on dendritic cells thereby regulating the functions of dendritic cells so as to treat diseases. For example, a peptide having a sequence of 5 to 8 amino acid residues of an extracellular region or an intracellular region of the receptor is suitable as an antigen

used in the preparation of an antibody. Specifically, for example, the fragmentary peptides used in Example 9 of the present specification, namely a fragmentary peptide consisting of the 6th to 32nd amino acid residues of the amino acid sequence of SEQ ID NO:2 and a fragmentary peptide consisting of the 1st to 23rd amino acid residues of the amino acid sequence of SEQ ID NO:2, can be used as antigens. As an example of fragmentary peptides used for screening a ligand, there can be mentioned a peptide having a sequence which is considered to be ligand-binding region(s) of C5L2. More specific examples of such fragmentary peptides include a peptide containing the N-terminal extracellular region (the 1st to 35th amino acid residues of SEQ ID NO:2), the 1st extracellular loop (the 96th to 108th amino acid residues of SEQ ID NO:2), the 2nd extracellular loop (the 172nd to 198th amino acid residues of SEQ ID NO:2), or the 3rd extracellular loop (the 681st to 726th amino acid residues of SEQ ID NO:2) of C5L2 protein.--.

Page 48, the paragraph beginning on line 5 has been amended as follows:

--The whole length nucleotide sequence of C5L2 obtained by the present inventors is shown in SEQ ID NO:1. A C5L2 clone having a nucleotide sequence which is different from that of SEQ ID NO:1 has also been identified. As a specific example of such clones, a C5L2 clone has been detected, which has a structure in

which a further thymine (t) is inserted to the [contiguous] sequence of six contiguous thymines at the site of the 724th to 729th nucleotides of SEQ ID NO:1 (that is, the C5L2 clone has a [contiguous] sequence of seven contiguous thymines at the site which substantially corresponds to the 724th to 729th nucleotides of SEQ ID NO:1). By the use of a nucleic acid probe or primer containing the above-mentioned site of C5L2 of the present invention (i.e., the site consisting of contiguous thymines), it is possible to detect separately a sequence having six contiguous thymines and a sequence having seven contiguous thymines.--.

Page 49, the paragraph beginning on line 15 has been amended as follows:

--Further, according to the present invention, there are also provided an isolated DNA and a derivative thereof, wherein the isolated DNA is a fragmentary[, contiguous] sequence of at least 12 contiguous nucleotides in the nucleotide sequence of SEQ ID NO:3 or 4; and an isolated RNA and a derivative thereof, wherein the isolated RNA is a fragmentary[, contiguous] sequence of at least 12 contiguous nucleotides in an RNA which is complementary to the nucleotide sequence of SEQ ID NO:3.--.

Page 50, the paragraph beginning on line 24 and bridging pages 50, 51 and 52 has been amended as follows:

--Examples of nucleic acid fragments useful for detecting a C5L2 cDNA or a genomic C5L2 DNA include a fragment

comprising a [contiguous] sequence of at least 12 contiguous nucleotides, preferably not less than 16 contiguous nucleotides, more preferably not less than 20 contiguous nucleotides, in the nucleotide sequence of SEQ ID NO:[2] 1 or 3, or in a DNA or an RNA which is complementary to the nucleotide sequence of SEQ ID NO:[2] 1 or 3. A derivative of the above-mentioned nucleic acid fragments can also be used. The length of the nucleic acid fragment may vary depending on the desired properties of the nucleic acid fragment, such as the specificity and the stability of binding to a nucleic acid to be detected. When PCR (Polymerase Chain Reaction) is conducted using a DNA fragment as a primer, it is preferred to use a DNA fragment having a T_m (melting temperature of DNA duplex) of 45 °C or more. In the PCR and the like where two DNA strands are bound to each other thereby forming a DNA duplex, the T_m of the DNA duplex can be estimated by calculating the sum of the temperature values assigned to GC pairs and AT pairs in the DNA duplex, wherein 4 °C is assigned to each GC pair and 2 °C is assigned to each AT pair. When a nucleotide sequence to be detected has a high GC content (90 % or more), a DNA fragment which is a [contiguous] sequence of at least 12 contiguous nucleotides can be used. Generally, the GC content of a nucleotide sequence is about 50 %. For detecting such a sequence, a DNA fragment which is a [contiguous] sequence of at least 16 contiguous nucleotides is needed. The

binding between a DNA and a nucleic acid derivative is more stable than the binding between two DNAs and, thus, when a nucleic acid derivative is used as a primer, it is possible to detect a desired DNA using a short nucleic acid sequence as compared to the case where a DNA is used as a primer.--.

Page 52, the paragraph beginning on line 8 and bridging pages 52 and 53 has been amended as follows:

--The examination of the ratio of the expression of the gene of the present invention for the purpose of diagnosis can be conducted by hybridization, primer extension, nuclease protection assay, reverse transcription PCR (RT-PCR) or the like in which a probe or primer designed based on the present invention is used. The probe and primer can be the DNA of SEQ ID NO:4 (i.e., antisense DNA having the sequence complementary to the DNA of SEQ ID NO:3), an RNA complementary to the DNA of SEQ ID NO:3 (i.e., antisense RNA), or a nucleic acid fragment which is a [contiguous] sequence of at least 12 contiguous nucleotides, preferably not less than 16 contiguous nucleotides, more preferably not less than 20 contiguous nucleotides in the above-mentioned DNA or RNA. The antisense DNA or RNA may be a methylated, methyl phosphorylated, deaminated or thiophosphorylated antisense nucleic acid derivative. For example, as shown in Example 4 described below, it is possible to detect the C5L2 mRNA using a fragment of the nucleotide sequence

of SEQ ID NO:4 (i.e., the sequence complementary to the DNA of SEQ ID NO:3).--.

Page 67, the paragraph beginning on line 12 and bridging pages 67 and 68 has been amended as follows:

--With respect to the antigen used for producing the antibody of the present invention, there is no particular limitation as long as the antigen has an amino acid sequence of a sufficient length for exhibiting the characteristics of the C5L2 protein. It is preferred that the antigen is a peptide which is a fragmentary[, contiguous] sequence of at least 5 contiguous amino acids in the amino acid sequence of SEQ ID NO:2, more advantageously at least 8 contiguous amino acids in the amino acid sequence of SEQ ID NO:2. The antigen peptide is used as such or after cross-linking the peptide with a carrier protein, such as KLH (Keyhole Limpet Hemocyanin) or BSA (bovine serum albumin). The antigen peptide (as such or in a form cross-linked with a carrier protein) is inoculated into an animal, wherein, if desired, an adjuvant may be administered together with the antigen peptide. Subsequently, from the animal, an antiserum containing an antibody (polyclonal antibody) recognizing the C5L2 protein can be obtained. The antiserum can be used as such. If desired, the antibody may be purified from the antiserum. Examples of animals into which the antigen peptide is inoculated include a sheep, a cattle, a goat, a rabbit, a mouse, a rat and

the like. For the preparation of a polyclonal antibody, the use of a sheep or a cattle is preferred. Specifically, as shown in Example 9 described below, there can be obtained an anti-human C5L2 protein rabbit polyclonal antibody and a solution of an anti-human C5L2 protein rabbit immunoglobulin.--.

Page 68, the paragraph beginning on line 16 and bridging pages 68 and 69 has been amended as follows:

--Further, a monoclonal antibody can be obtained by a conventional method for producing a hybridoma cell. For the production of a monoclonal antibody, it is preferred to use a mouse. As an antigen peptide, there may be used a fusion protein which is obtained by linking GST (glutathione S-transferase) to an antigen peptide which is a fragmentary[, contiguous] sequence of at least 5 contiguous amino acids in the amino acid sequence of SEQ ID NO:2, preferably at least 8 contiguous amino acids in the amino acid sequence of SEQ ID NO:2. The fusion protein may be either a purified one or a non-purified one. Also, a monoclonal antibody can be obtained by using a gene recombinant antibody which has been expressed in a cell by using an immunoglobulin gene which has been separated by using various methods described in a reference book ("Antibodies a laboratory manual", E. Harlow et al., Cold Spring Harbor Laboratory) and using a gene cloning method.--.

Page 82, the paragraph beginning on line 4 and bridging pages 82 and 83 has been amended as follows:

--Examples of primers used for the RT-PCR method include the DNA fragments of the present invention and derivatives thereof, wherein the DNA fragments are fragmentary[, contiguous] sequences (each independently being a [contiguous] sequence of at least 12 contiguous nucleotides, preferably not less than 16 contiguous nucleotides, more preferably not less than 20 contiguous nucleotides) in the nucleotide sequences of SEQ ID NOs:3 and 4, respectively. Specific examples of the primers include synthetic primers shown in SEQ ID NOs:9 and 10. The amount of the mRNA encoding C5L2 can be measured by the method described in Example 11 of the present specification. More specifically, the amount of the mRNA encoding C5L2 protein can be measured by a method in which the mRNA encoding the receptor C5L2 in a sample is detected by using the primers shown in SEQ ID NOs:9 and 10, whereas the mRNA encoding glyceraldehyde 3-phosphate dehydrogenase (G3PDH) in the same sample as mentioned above is detected by using the primers shown in SEQ ID NOs:11 and 12. The amount of the mRNA encoding C5L2 protein is determined in terms of the ratio of the amount of the mRNA based on the amount of the PCR product for G3PDH (i.e., ratio of the expression of C5L2 based on the expression of G3PDH).--.

Page 83, the paragraph beginning on line 3 and bridging pages 83 and 84 has been amended as follows:

--In the present invention, there is no particular limitation with respect to the method for measuring the amount of the C5L2 protein present on the cell surface as long as the amount of the C5L2 protein can be measured specifically. For specifically measuring the amount of the C5L2 protein, it is preferred to use an antibody which specifically binds to C5L2 receptor. Examples of such antibodies include an antibody prepared in Example 9 of the present specification, which is prepared using, as an antigen, a peptide which is a fragmentary[, contiguous] sequence of at least 5 contiguous amino acid residues in the amino acid sequence of SEQ ID NO:2. Examples of methods for measuring the amount of the C5L2 protein using the above-mentioned antibody include FACS (employed in Example 11 of the present specification) and immunoprecipitation. With respect to examples of clinical diagnosis using FACS, reference can be made to a text book, such as "Furo-Saitometorii-Handobukku (Flow Cytometry Handbook)", Yoshio TENJIN et al. eds. (published in 1984 by SCIENCE FORUM INC., Japan), particularly to Section 4: "Furo-Saitometorii-no Rinsho-igaku-eno Ouyo (Application of Flow Cytometry to Clinical Medicine)" thereof. With respect to the methods for conducting immunoprecipitation and immunoassay, reference can be, respectively, made to pages 421 to 470 and

pages 553 to 612 of "Antibodies a laboratory manual" (E. Harlow et al., Cold Spring Harbor Laboratory).--.

IN THE CLAIMS:

Claim 1 has been amended as follows:

--1. (amended) A [substantially pure] purified human seven-pass transmembrane receptor protein having the amino acid sequence of SEQ ID NO:2.--

Claim 2 has been amended as follows:

--2. (amended) A [substantially pure] purified peptide which is a fragmentary[, contiguous] sequence [of at least 5 amino acids in the amino acid sequence of SEQ ID NO:2] selected from the group consisting of the 6th to 32nd amino acid residues of SEQ ID NO:2, the 1st to 23rd amino acid residues of SEQ ID NO:2, the 1st to 35th amino acid residues of SEQ ID NO:2, the 96th to 108th amino acid residues of SEQ ID NO:2, the 172nd to 198th amino acid residues of SEQ ID NO:2, and the 681st to 726th amino acid residues of SEQ ID NO:2.--

Claim 5 has been amended as follows:

--5. (amended) An isolated DNA or a chemically modified nucleic acid derivative thereof, wherein said isolated DNA is a fragmentary[, contiguous] sequence of at least [12] 20 contiguous nucleotides in the nucleotide sequence of SEQ ID NO:3.--

Claim 6 has been amended as follows:

--6. (amended) An isolated DNA or a chemically modified nucleic acid derivative thereof, wherein said isolated DNA is a fragmentary[, contiguous] sequence of at least [12] 20 contiguous nucleotides in the nucleotide sequence of SEQ ID NO:4.--

Claim 7 has been amended as follows:

--7. (amended) An isolated RNA or a chemically modified nucleic acid derivative thereof, wherein said isolated RNA is a fragmentary[, contiguous] sequence of at least [12] 20 contiguous nucleotides in an RNA which is complementary to the nucleotide sequence of SEQ ID NO:3.--

Claim 8 has been amended as follows:

--8. (amended) A replicable recombinant DNA, comprising a replicable expression vector and, operably inserted therein, the isolated DNA according to [one of claims 3 to 6] any one of claims 3, 4, 5 or 6.--

Claim 10 has been amended as follows:

--10. (amended) A seven-pass transmembrane receptor protein [obtainable] prepared by a process which comprises:

(a) ligating, to a replicable expression vector, the isolated DNA according to claim 3 or 4, to thereby obtain a replicable recombinant DNA having said replicable expression vector and, operably inserted therein, said DNA;

(b) transforming cells of a microorganism or cell

culture with said replicable recombinant DNA to form transformants;

(c) selecting said transformants from parent cells of the microorganism or cell culture; and

(d) culturing said transformants, causing said transformants to express said DNA and produce a protein on the cell surface of said transformants.--

Claim 24 has been amended as follows:

--24. (amended) A method for screening a substance which inhibits a ligand from binding to the seven-pass transmembrane receptor peptide of claim 2, which comprises:

contacting [the] said peptide [of claim 2, or the peptide of claim 2] with a ligand which binds to [said protein or] said peptide and a sample which is suspected to contain a substance which inhibits said ligand from binding to [said protein or] said peptide;

assessing a change occurring in response to a binding of said ligand to [said protein or] said peptide; and

detecting said substance by using said change as an index.--

Exhibit 1

ANTISENSE RNA AND DNA

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Antisense RNA and DNA: 1–49

Antisense Techniques: An Overview

James A.H. Murray and Nigel Crockett

I. INTRODUCTION

It is indeed a paradox that it is possible to isolate virtually any gene from any desired organism and to establish its DNA sequence and expression pattern in great detail, yet there exist very few techniques that allow us to approach its in vivo function. The amount of DNA sequence data is accumulating rapidly, making the problem of determining the biochemical and developmental roles of the open reading frames identified ever more acute. Antisense methods are an important tool in this process, since, analogous to the use of inhibitors for studies of proteins, they provide a method of preventing or reducing gene function by targeting the genetic material or its expression. Their application requires only a knowledge of the DNA or RNA sequence of the target gene.

The realization of the genetic code by gene expression requires an information flow from gene to protein. This depends at each stage on specific base pairing between complementary nucleic acids to ensure the accuracy of transmission and interpretation of the information, and herein lies the basis for antisense methods. Our understanding of the molecular details of these processes has developed from the model of the complementary nature of the two strands of the double helix proposed by Watson and Crick to an appreciation that both single-stranded DNA and RNA can form homo- and heterodimers of DNA:DNA, RNA:DNA, and RNA:RNA hybrids and that such complementary interactions are of intrinsic importance to transcription, RNA processing, and translation.

The fundamental importance of specific base pairing to the function of nucleic acids offers the possibility of interfering with the expression of target genes in a highly selective manner by using a complementary or *antisense* sequence. In general terms, the antisense sequence will hybridize to its target and block expression by one of several possible means. It may act by preventing other complementary interactions from occurring that are necessary for expression or occluding a process that requires a single-stranded substrate, by stimulating the action of nucleases specific for the double-stranded regions of DNA:RNA or RNA:RNA hybrids, or in certain cases by inactivating the target directly by intercalation or cleavage. It should therefore be apparent that antisense strategies can in principle be used for any gene for which sequence information is available and in any system into which antisense nucleic acid can be introduced by external application, by microinjection, or by nuclear expression from a construct containing an artificial antisense gene.

Thus the ultimate objective of the antisense approach is to use a sequence complementary to the target gene to block its expression and thereby create a mutant cell line or organism in which the level of a single chosen protein (or, in certain cases, the replication of a virus) is selectively reduced or abolished. The aim may be to produce a phenotype to investigate the function of the gene in developmental or cellular processes, to reduce expression of a gene causing an undesirable phenotype in transgenic plants or animals, or to interfere with oncogenic transformation or viral infection. The antisense approach is clearly particularly attractive for those eukaryotic organisms that do not have well-defined or amenable genetic systems because of long generation time, genome complexity, or difficulty in isolating recessive mutations in diploids. In contrast, anti-

sense techniques enable a "phenocopy" of the gene mutation to be produced using only the cloned sequences. However, as will become clear, even in classic genetic organisms such as *Drosophila* antisense methodology can be a convenient short-cut to answering certain types of question.

Other "reverse genetic" approaches besides antisense are also available for assisting in the assignation of function of a cloned gene. The most clear-cut and effective way to extinguish the function of a gene is to delete or disrupt its coding sequences in a heritable way by homologous recombination. However, this approach is laborious, not least because both alleles of a gene must be removed and it is at present restricted to a few organisms [yeast, Winston et al., 1983; *Dictyostelium*, De-Lozanne and Spudich, 1987; plants, Paszkowski et al., 1988; Lee et al., 1990; mice; Schwartzberg et al., 1989; and human cells, Song et al., 1987]. Ectopic expression, in which a gene is introduced back into cells or an organism under a heterologous promoter to cause alteration in the temporal or spatial pattern of its expression, and overexpression are alternative approaches that share with antisense a possible dominant phenotype, exhibited despite the presence of wild-type copies of the gene. There are, however, many gene products that for a number of reasons can show no phenotype when their level is increased. A way around this problem involves the fact that it is sometimes feasible to overexpress a mutant form of a gene whose product acts as part of a complex so that it competes with the wild-type protein and disrupts the normal activity of the complex to produce a dominant negative phenotype.

In comparison, antisense is broadly available and applicable and has the important advantage that a range of phenotypes can be produced corresponding to various levels of expression. The site of inactivation and its developmental effect can be manipulated by the choice of promoter for antisense genes or by the timing of external application or microinjection. This is particularly an advantage for genes with an essential cellular or developmental function or when it is desirable to examine the role of a gene in a particular subset of cells in an organism. A further feature of the antisense approach is that it is possible to manipulate its specificity by selecting either unique regions of the target gene or regions where it shares homology to other related genes and thereby investigate the role of functions encoded by gene families. It is, however, important to realize that antisense strategies have not been as universally straightforward or as easy to apply as was initially hoped, nor has the interpretation of results always been unambiguous, and this has perhaps led to their premature dismissal in certain instances. Many chapters in this book, and the millions of dollars currently being invested in the biotechnological application of antisense technology [Klausner, 1990], bear witness to the claim that antisense approaches can be successful. Now that the initial euphoria and subsequent disenchantment has given way to a greater pragmatism, it is clear that there is an important future for antisense approaches not only as a research tool but also in biotechnology for manipulating the characteristics of transgenic animals and plants and in pharmacology as therapeutic agents [Uhlmann and Peyman, 1990]. It is the purpose of this book to draw together the practical lessons from a wide range of systems, with the successes and failures, to provide not only an overview of the field but also a source of relevant practical information on which to build future experiments.

In this overview, we follow this discussion of the general principles that lie behind the antisense dogma with an outline of the classes of antisense agents available and how they are applied. We then go on to consider in more detail evidence for the mechanisms of antisense regulation, since one of the least satisfactory and satisfying aspects of antisense is the lack of consistent and generally applicable information on its precise mode of action, which makes a rational design of antisense strategies difficult. This is partly due to the variety of different agents used, partly to a dearth of systematic data relating to questions of mechanism, but

is perhaps chiefly a reflection of the large number of variables within the multistep process from gene to protein. Any one of these may or may not be a target, depending on mRNA structure, accessibility, transport, and other factors that can differ from gene to gene, cell type to cell type, and organism to organism.

After attempting to draw some conclusions on mechanism, we switch attention exclusively to antisense RNA and discuss practical considerations of its use and possible reasons for failure. The chapter concludes with a brief survey of the use of antisense RNA in various eukaryotic systems, concentrating on those not covered in pages 77–174.

II. ANTISENSE TOOLS

The materials with which the antisense approach can be applied are broadly divided into antisense RNA, antisense DNA oligomers, and catalytic RNAs or ribozymes. In this section we briefly review the features of each class of reagent, and, since the choice of the type of nucleotide sequence to be used as the antisense reagent is inevitably a compromise, we concentrate on the characteristics that are most important in the consideration of how each approach can be used. Figure 1 summarizes the various methods available for the introduction of antisense reagents into cells. We also point the reader to other chapters in the book where various points are covered in much greater detail. In addition, a considerable number of reviews have appeared that cover aspects of antisense regulation, and these are summarized in Table 1.

A. Enabling Technologies

In addition to methods for establishing the DNA sequence of genes and for introducing vectors carrying new genetic information back into cells and organisms, two important developments were required for the application of certain antisense techniques. The first of these was the discovery that purified RNA polymerases encoded by bacteriophages such as Sp6, T7, and T3 can be used in vitro to produce large quantities of specific RNA by cloning the desired DNA sequence downstream of the appropriate promoter [Melton et al., 1984; Davanloo et al., 1984]. Since these RNA polymerases will transcribe on a linear molecule, the template can be cut with a convenient restriction endonuclease to define the 3′ end of the RNA to be synthesized, and run-off transcription will then produce RNAs of a defined length. The ability to synthesize RNA efficiently in vitro is important not only for microinjection of antisense RNAs but also for producing radiolabeled strand-specific probes that can be used to detect independently the presence of sense and antisense transcripts in cells by Northern blotting or nuclease mapping techniques.

The second advance, of importance for strategies using oligonucleotides, is the increased efficiency and availability of automated oligonucleotide synthesizers. Together with improvements in synthetic chemistry this now allows a much greater range of analogs to be produced by manipulation of the procedures. Readers are referred to other sources for details of oligonucleotide synthesis [Gait, 1984; Uhlmann and Peyman, 1990].

B. Antisense RNA

In the early 1980s, the realization that natural antisense RNA is involved in the regulation of plasmid copy number in bacteria was followed by the discovery of a considerable number of prokaryotic examples in which antisense RNA is implicated in regulation of plasmid and phage systems and in the expression of certain chromosomal genes. This then led to the development of artificial antisense RNA regulatory systems, both in *Escherichia coli* and in eukaryotes. Antisense RNA in prokaryotes is reviewed in detail by Thomas (this volume).

1. Nuclear expression of RNA by engineered antisense genes. The principle of regulation by antisense RNA is extremely simple in that RNA that is complementary to the target mRNA is introduced into cells, resulting in specific RNA:RNA duplexes being formed

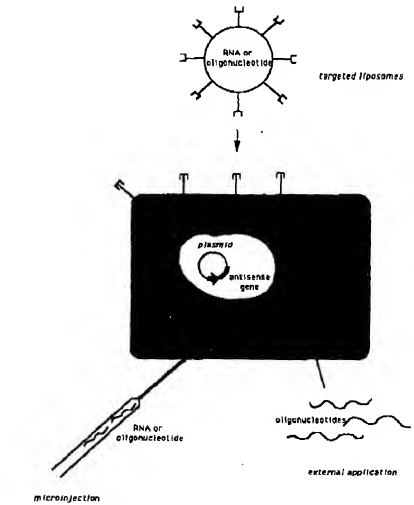


Fig. 1. *Approaches that can be used to introduce antisense nucleic acids into cells are expression from antisense gene constructs introduced in a transient or stable system; microinjection of RNA or oligonucleotides; liposomes carrying RNA or oligonucleotides and targeted with antibodies; and addition of oligonucleotides to cell culture medium.*

by base pairing between the antisense substrate and the target mRNA.

This can be achieved in vivo by the introduction and expression of an antisense gene sequence, that is, one in which part or all of the normal gene sequences are placed under a promoter in inverted orientation so that the "wrong" (complementary) strand is transcribed into a noncoding antisense RNA that then hybridizes with the target mRNA and interferes with its expression. Such an antisense expression vector can be constructed by standard procedures and introduced into cells by any of the normal means of transferring DNA, such as transfection, electroporation, microinjection, or, in the case of viral vectors, by infection. The type of transformation and choice of vector will determine whether expression is transient or stable, while the promoter used for the antisense gene will influence the level, timing, inducibility, or tissue specificity of the antisense inhibition.

TABLE I. Published Reviews on Antisense RNA and DNA, Catalytic RNA, and Related Areas

Authors	Antisense topic	Notes
Books		
Wickstrom [1991]	Prospects for antisense therapy	Regulation of viruses and oncogenesis
Cohen [1989a]	Antisense oligonucleotides	
Melton [1988]	Antisense RNA and DNA	Papers presented at the 1987 Banbury Meeting, Cold Spring Harbor
Review articles		
General		
Colman [1990]	Antisense RNA and DNA	Review of applications in cell and developmental biology
Hélène and Toulmé [1990]	Antisense RNA and DNA	Useful general review
Holt and Lechner [1990]	Antisense RNA and DNA	Short discussion of practical uses
Weintraub [1990]	Antisense RNA and DNA	*Scientific American* review
Walder [1988]	Antisense RNA and DNA	Short commentary on prospects
Van der Krol et al. [1988b]	Antisense RNA and DNA	Useful general review
Antisense RNA		
Mol et al. [1990]	Antisense RNA in plants	Short review
Takayama and Inouye [1990]	Antisense RNA	Detailed and useful review
Hiatt et al. [1989]	Antisense RNA in plants	Application in tomato of antisense PG
Izant [1989]	Antisense RNA	Applications in cytoskeleton
Kreis [1989]	Antisense RNA	Application to cytoskeleton studies in *Dictyostelium*
Inouye [1988]	Antisense RNA in prokaryotes	Discusses design of artificial antisense
Simons [1988]	Natural antisense RNA	Brief review of bacterial systems
Simons and Kleckner [1988]	Antisense RNA in prokaryotes	Naturally occurring systems and mechanisms
Van der Krol et al. [1988a]	Antisense RNA in plants	Brief review
Green et al. [1986]	Antisense RNA	Review of prokaryotic and eukaryotic gene regulation
Weintraub et al. [1985]	Antisense RNA	Short discussion of early results
Antisense DNA		
Miller [1991]	Antisense methylphosphonates	Review
Delnick [1990]	Antisense oligonucleotides	Short review of pharmacological applications
Sherman [1990]	Antiviral oligonucleotides	Short discussion of relevant considerations
Sigman and Chen [1990]	Chemical nucleases	Chemical cleavage agents and site-specific nucleases
Uhlmann and Peyman [1990]	Antisense oligonucleotides	Detailed and comprehensive review of all aspects
Zon [1990]	Antiviral oligonucleotides	Short discussion of anti-HIV potential
Cohen [1989]	Antisense oligonucleotides	Pharmaceutical possibilities
Stein and Cohen [1989]	Antisense oligonucleotides	Review of potential role in cancer therapy
Loose-Mitchell [1988]	Antisense oligonucleotides	Brief discussion of pharmaceutical possibilities
Marcus-Sekura [1988]	Antisense oligonucleotides	Practical approaches
Stein and Cohen [1988]	Antisense oligonucleotides	General review
Toulmé and Hélène [1988]	Antisense oligonucleotides	Short review

TABLE I. Published Reviews on Antisense RNA and DNA, Catalytic RNA, and Related Areas (*Continued*)

Authors	Antisense topic	Notes
Ribozymes and catalytic RNA		
Cech [1990]	Self-splicing of group I introns	Full review of structure, reactions, and chemistry; especially *Tetrahymena* intron
Perriman and Gerlach [1990]	Ribozyme technology	Brief survey of recent work on all ribozyme types
Rossi and Sarver [1990]	Ribozymes as antiviral agents	Review of *trans*-acting ribozymes with potential intracellular uses
Symons [1989b]	Self-cleaving pathogenic RNAs	Viruses and virusoids; hammerhead structures
Michel et al. [1989]	Group II catalytic introns	Conserved sequence and structure
Cech [1988]	Group I introns	Conserved sequence and structure
Cech [1987]	Chemistry of RNA enzymes	Mechanistic and enzymatic considerations
Cech and Bass [1986]	Catalytic RNA	General review
Waugh and Pace [1985]	Catalytic RNA	*Tetrahymena* intron and RNase P

Such artificial antisense genes have been demonstrated to regulate the expression of both endogenous and introduced target genes and have therefore found broad application in an impressive range of prokaryotic and eukaryotic systems, as discussed in Section V and elsewhere in this book, showing how biochemical and developmental functions can be targeted to answer questions about their biological role.

2. Microinjection of in vitro transcribed RNA. An alternative approach to antisense genes makes use of purified phage polymerase to produce RNA with subsequent microinjection of the in vitro transcribed antisense transcripts. Clearly some systems such as *Drosophila* (see Patel and Jacobs-Lorena, this volume), the mouse embryo (Levy et al., this volume), and *Xenopus* (see chapters by Bass and by Colman, this volume) are more amenable to this approach than others, because of the difficulty of injecting or accessing certain cells, and it is normally more laborious than using antisense genes. Application in multicellular organisms is normally difficult, being restricted in the case of *Drosophila*, for example, to the period of embryogenesis before cellularization occurs. However, unlike the antisense gene approach, microinjection is not restricted by the availability of suitable promoters or vectors, and it may be the only approach for cells that are transcriptionally inactive. Indeed, a major advantage is that a much larger excess of antisense RNA over target can be injected than can normally be expressed from endogenous antisense genes, although this is compensated for somewhat because of the relatively lower efficiency of injected antisense RNA in inhibiting gene expression. This is the consequence of a more subtle difference between the two approaches, in that RNA is normally injected into the cytoplasm and thus any potential points of antisense regulation within the nucleus are missed. It can therefore be concluded that duplex formation by injected antisense RNA must act by preventing translation or by provoking attack by double-stranded specific RNases. We return to this point in Section III.

The most obvious difference between injected RNA and nuclear antisense expression is that the effect of injection will of necessity be transient, as the RNAs are degraded or diluted during growth. This can, however, be used to advantage in certain situations, since by injecting the antisense RNA, and therefore ablating the gene function, at a specific time, it may be possible to show that the gene has a key role within a particular window of development.

3. Delivery of antisense RNA by liposome fusion. A recent approach that may be useful for both in vitro and in vivo delivery of antisense reagents, including RNA, is the use of liposomes. Encapsulation int liposomes protects

RNA (and DNA) against degradation by nucleases. Liposomes may be targeted to certain cells by coupled antibodies that ensure specific interaction with cells that carry corresponding surface antigens into which they are taken up by receptor-mediated endocytosis. This is discussed further in the chapters by Degols et al. (Section II.D) and by To (Section III.B.3), this volume.

C. Antisense DNA: Inhibition by Oligodeoxynucleotides and Their Derivatives

Antisense oligodeoxynucleotides are short sequences of single-stranded DNA, usually less than 30 nucleotides (nt) in length, synthesized by chemical means in vitro, and are complementary to a specific intracellular target, normally mRNA. More recently, oligonucleotides designed to form triple helices with double-stranded DNA, or selected to interact with proteins, have been developed, and these are mentioned below. The general use of oligonucleotides and their analogs to modulate gene expression is referred to as *antisense DNA* regulation in this book, as distinct from the use of antisense genes for nuclear expression of *antisense RNA*. The whole subject of gene regulation by antisense DNA is reviewed in detail in this volume by Toulmé, and specific examples are given in the chapters following that (see also Table I), so here we will discuss only general principles.

Because antisense oligonucleotides are produced by chemical synthesis, they must be applied either externally to cells or microinjected (see Fig. 1), and as a consequence their effects will normally be transient. However, they offer the important advantage that the full power of synthetic organic chemistry can be used to design oligomers with particular modified linkages or terminal groups, which results in improved properties. It is on such modified oligonucleotides that most hope is pinned for developing antisense therapeutics for human antiviral or anticancer treatment, and such work is the subject of several chapters in this book.

1. Unmodified phosphodiester oligodeoxynucleotides: "n-oligos." It was with short DNA oligonucleotides rather than with RNA that the first demonstrations of an antisense effect were made, initially using in vitro translation systems (see Toulmé, this volume, for references), and subsequently demonstrating direct inhibition of Rous sarcoma virus (RSV) replication in cultured cell lines by synthetic oligonucleotides added to the culture medium [Zamecnik and Stephenson, 1978]. Subsequent experiments have shown that inhibition of various viruses (see chapters by To and by Agrawal and Leiter) and the expression of genes (see particularly Wickstrom's chapter) can be achieved, clearly indicating that unmodified oligonucleotides ("n-oligos") are internalized by cells and can interact with intracellular targets. However, it is a common observation that n-oligos are rapidly attacked and degraded by nucleases present in the serum component of culture medium, which can only be partly overcome by heat inactivation (see chapters by Toulmé (Section III.B); Tidd; Matsukura; and Wickstrom) [Holt et al., 1988]. Moreover, after uptake, oligonucleotides are subject to continuing degradation within cells, though at a greatly reduced rate compared with their attack in the culture medium.

It was in fact surprising that n-oligos can have an effect simply by adding them to the cell culture medium, since it was not expected that such highly charge molecules could cross the plasma membrane. It is now generally believed that their uptake occurs by receptor-mediated endocytosis (see discussion by Tidd [Section IV], this volume), but it is clearly not a very efficient process. Wickstrom (this volume) calculates that only 1%–2% of added oligonucleotides were internalized by cells after 4 hours, but gel electrophoresis showed that after uptake oligonucleotides were relatively stable, reducing to 25% of their original level after 24 hours. In contrast, degradation of oligonucleotides in the culture medium was complete after 8 hours. It is primarily in search of solutions to these problems of poor uptake and nucleolytic attack that derivatives of oligonucleotides that would be more stable and lipid soluble were sought.

2. Oligonucleotide analogs with backbone modificati ns. The dual aims of improving

both cell uptake and resistance to nucleases were tackled by synthesizing derivatives with substitutions in place of one of the nonbridging oxygens in the internucleotide bond of the backbone. In particular, methylphosphonates, where $-CH_3$ replaces the $-O$ (discussed by Miller, this volume), and phosphorothioate (where $-S$ replaces $-O$, abbreviated to "S-oligos") have been explored (see Matsukura, this volume), both of which show greater extra- and intracellular longevity because of increased nuclease resistance. These and other modified oligos are shown in Figure 2. The elimination of the negative charge in the internucleotide phosphate bridge in methylphosphonates considerably enhances cellular uptake, probably by improving lipid solubility, and it appears that such oligonucleotides enter cells slowly until the intracellular and extracellular concentrations are approximately equal, presumably by simple diffusion through the membrane. However, in contrast, phosphorothioate oligonucleotides do not penetrate cells efficiently, requiring their use at relatively high concentrations.

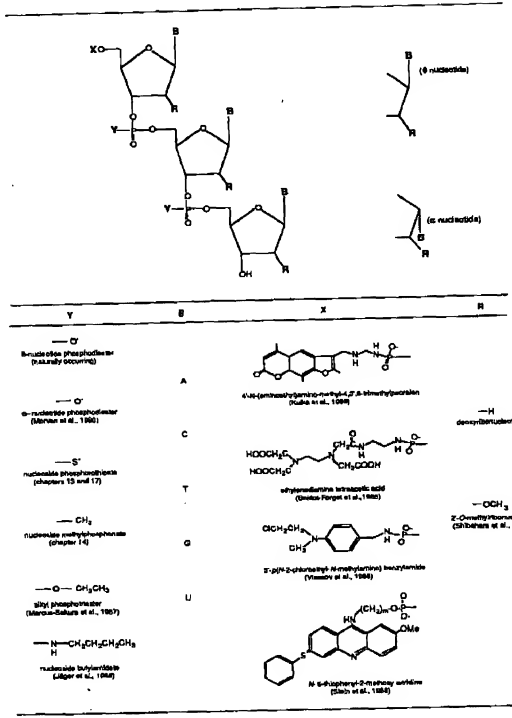
Of particular relevance in a consideration of the use of analogs is their mechanism of antisense action. This is discussed in greater detail in Section III, but it is necessary to appreciate here that unmodified n-oligos mediate their effects because they stimulate the cleavage of their target RNA by RNase H, which cuts the RNA component of RNA:DNA duplexes. Phosphorothioate oligonucleotide:RNA duplexes are also substrates for RNase H, whereas methylphosphonate oligonucleotide:RNA duplexes are not, and must therefore act as physical blocks that prevent splicing or translation machinery from accessing the RNA. A promising approach, which is discussed by Tidd (this volume), uses "sandwich" oligonucleotides of a central core of nucleotide units linked by normal phosphodiester bonds, protected from 5′ and 3′ exonucleolytic attack by terminal nucleotides linked by methylphosphonate bonds.

A second type of backbone modification is exhibited by the α-oligomers, which have an unnatural glycosidic configuration (Fig. 2) that results in nuclease resistance and in an increase in the temperature of dissociation (T_m) of the RNA:α-DNA duplex and therefore an improvement in the stability of the hybrid. Although not a substrate for RNase H, they can inhibit translation when targeted at the cap site of an mRNA [Boiziau et al., 1991].

3. Terminally modified oligonucleotides. The alternative route to improving oligonucleotide efficiency is by attaching terminal groups. This may again be aimed at improving their membrane penetration, for which 3′-conjugated groups such as polylysine and cholesterol have been used (see Degols et al., this volume), or they may be designed to increase oligonucleotide reactivity. This latter type of modification is reviewed by Toulmé (this volume) and includes acridine rings (which increase the affinity toward RNA), alkylating and cross-linking moieties (psoralen), and metal complexes that can cleave the RNA target (Fig. 2). Acridine has the additional advantages of improving cell uptake and of allowing this to be followed by the green fluorescence it produces. An example of its effective use in an oligonucleotide targeted against the common 5′ end of mRNAs for variant surface glycoproteins in the unicellular flagellate parasite *Trypanosoma brucei* is given by Verspieren et al. [1987].

It will be clear from this brief discussion that there are many backbone and terminal modifications to oligonucleotides that might improve their performance. Cohen [1989b] points out that 15 classes of compound can be synthesized simply by substituting each of the four groups attached to the phosphorus in the internucleotide bond with O–, CH_3, or S– in various combinations. Many of these have not been synthesized, and fewer have been tried with additional terminal groups.

Although there are signs that a more systematic approach toward combining the advantages of various types of analog is emerging, such as the methylphosphonate–phosphodiester chimeras (see Tidd, this volume), it is true that no clear generalizations have emerged on the use of anti-mRNA oligonucleotides to date. This is not to imply that notable successes in gene


(β nucleotide)
(α nucleotide)
Y
B
X
R
—O⁻
S-nucleoside phosphodiester (naturally occurring)
—O⁻
O-nucleoside phosphodiester (Michelson et al., 1960)
—S⁻
nucleoside phosphorothioate (chapters 15 and 17)
—CH₂
nucleoside methylphosphonate (chapter 14)
—O—CH₂CH₃
alkyl phosphotriester (Marcus-Sekura et al., 1987)
—N—CH₂CH₂CH₂CH₃ H
nucleoside butylamidate (Jäger et al., 1984)
A
C
T
G
U
4-(4-(aminoethyl)amino-methyl-4,5'-8-trimethylpsoralen (Hudka et al., 1988)
ethylenediamine tetraacetic acid (Boutes-Forget et al., 1982)
2'-p[(N-2-chloroethyl-N-methylamino) benzylamide (Vlassov et al., 1986)
N-5-Pridinyl-2-methoxy sulfilimine (Dietz et al., 1984)
—H
deoxyribonucleotide
—OCH₃
2'-O-methylribonucleotide (Shibahara et al., 1989)

regulation have not been observed, since this is clearly the case in many chapters in this book and elsewhere, but that shortcomings in the experimental systems often do not allow the modes of action, specificity, and toxicity to be thoroughly studied. It is in the rabbit reticulocyte cell-free system and in *Xenopus* (see Minshull and Hunt, this volume; Colman, this volume) that most systematic analysis is probably possible, and the reader is particularly pointed to the discussions by Minshull and Hunt (this volume) on specificity and interpretation of results.

It is clear that the in vivo use of oligonucleotides as potential therapeutic agents will require further improvements in cell uptake to reduce nonspecific toxicity effects and the cost per dose [Geiser, 1990]. The possibility of using liposomes as a delivery system, as mentioned above for RNA [see Degols et al., this volume; Pidgeon et al., 1990], offers great potential in vivo advantages of protection from nucleases as well as targeting to specific cells by antibodies. However, a further problem here is the short half-life of conventional liposomes in serum caused by uptake into the reticuloendothelial system (liver and spleen), although this could perhaps be alleviated by newer generations of liposomes such as those described by Gabizon and Papahadjopoulos [1988].

Despite the manifest problems, there is increasing interest in the pharmacological potential of oligonucleotides [Zon, 1990] (see references in Table I), accompanied by considerable commercial investment [Klausner, 1990]. Because costs are falling [Geiser, 1990], antisense treatments may soon be tried against life-threatening viral infections such as AIDS.

4. Triple helix-forming oligonucleotides. There has been recent excitement [Riordan and Martin, 1991; Charles, 1991] about the possibility of interfering directly with gene expression or viral replication by targeting DNA with oligonucleotides that bind in a sequence-specific manner in the major groove of a double-stranded target to form a triple helix or triplex structure (Fig. 3a). Such "anti-gene" oligonucleotides make use of the fact that recognition sites still remain in the major and minor grooves of the Watson-Crick double-stranded DNA structure. In particular, it is possible for thymine to form hydrogen bonds with adenine, while the adenine is simultaneously involved in Watson-Crick bonding with thymine (Fig. 3b). Similarly, protonated cytosine can bind to the guanine of a G.C base pair. These patterns of hydrogen bonding are referred to as *Hoogsteen base pairing*. As a consequence, a pyrimidine (cytosine and thymine) oligonucleotide can form a triple helix structure within the major groove of a purine (adenine and guanine) stretch of a double-stranded DNA molecule (Fig. 3a), with specificity provided by Hoogsteen base pairing. Such structures can form on supercoiled and relaxed DNA targets [Maher et al., 1989]. However, the binding is pH dependent, the triple helical structures with C or T on the Hoogsteen strand being stable in acid conditions but, because of the requirement for the cytosine to be protonated to bind to a G.C base pair, dissociating with increasing pH. Povsic and Dervan [1989] have shown that triple helix formation can be stabilized and extended to physiological pH by substituting the analog 5-bromouracil and/or 5-methylcytosine for thymine and cytosine, respectively. An example of the type of triple helix-forming oligonucleotide that can be used is shown in Figure 3c.

The potential advantage of the triple helix over the classic antisense approach for regulating genes lies in the enormously reduced number of molecules per cell that must be inactivated to inhibit expression if the DNA of the

Fig. 2. *Generic structure of antisense DNA oligomer (top) and an indication of the range of analogs that have been tested for antisense regulatory activity (bottom). B, base (adenine, cytosine, thymine, guanine, or uracil) through which hydrogen bonding to the target occurs; Y, different substituents on the phosphorous atom in the nucleotide phosphodiester linkage in place of the naturally occurring oxygen; X, different 5' terminal groups that can in principle be used with any internucleoside linkage; R, normally hydrogen, but the use of a 2'-O-methylribonucleoside has also been reported. A full review has been published by Uhlmann and Peyman [1990].*